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Attorney's Docket 037003-0269728
Client Reference: 2000-30-0613A

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re PATENT APPLICATION of: Confirmation No: 7677

Mitchell E. REFF

Application No.: 09/982,649 Group Art Unit: 1644

Filed: October 22, 2001 Examiner: Ronald Schnizdron

Title: VARIANT IGG3 RITUXAN AND THERAPEUTIC USE THEREOF

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(2) Response to Notice to Comply
(3) Copy of Human et al. journal article
(4) Preliminary Amendment

PILLSBURY WINTHROP LLP

THOMAS A. CAWLEY, JR., PH.D.
Reg. No. 40944

Date: October 26, 2004
P.O. Box 10500
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(Certification of Facsimile Transmission--page 1)

Attorney's Docket 037003-0280728
Client Reference: 2000-30-0513A

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re PATENT APPLICATION of: Confirmation No: 7677

Mitchell E. REFF

Application No.: 09/982,849 Group Art Unit: 1644

Filed: October 22, 2001 Examiner: Ronald Schwadron

Title: VARIANT IGG3 RITUXAN AND THERAPEUTIC USE THEREOF

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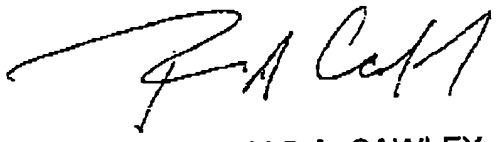

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
09/982,849	10/22/2001	Mitchell E. Reff	P 0280728 2000-30-0513A	7677

909 7590 10/06/2004

PILLSBURY WINTHROP, LLP
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EXAMINER	
SCHWADRON, RONALD B	

ART UNIT	PAPER NUMBER
1644	

DATE MAILED: 10/06/2004

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PTO-90C (Rev. 10/03)

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APPLICATION NO./ CONTROL NO.	FILING DATE	FIRST NAMED INVENTOR / PATENT IN REEXAMINATION	ATTORNEY DOCKET NO.

EXAMINER

ART UNIT	PAPER
	200410

DATE MAILED:

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This application contains sequence disclosures that are encompassed by the definitions for nucleotide and/or amino acid sequences set forth in 37 CFR 1.821(a)(1) and (a)(2). However, this application fails to comply with the requirements of 37 CFR 1.821 through 1.825 for the reason(s) set forth below or on the attached Notice To Comply With Requirements For Patent Applications Containing Nucleotide Sequence And/Or Amino Acid Sequence Disclosures.

No sequence listing has been submitted for the sequence PSAG disclosed on page 25 of the specification.

Applicant is given ONE MONTH, or THIRTY DAYS, whichever is longer, from the mailing date of this letter within which to comply with the sequence rules, 37 CFR 1.821 - 1.825. Failure to comply with these requirements will result in ABANDONMENT of the application under 37 CFR 1.821(g). Extensions of time may be obtained by filing a petition accompanied by the extension fee under the provisions of 37 CFR 1.136(a). In no case may an applicant extend the period for reply beyond the SIX MONTH statutory period. Direct the reply to the undersigned. Applicant is requested to return a copy of the attached Notice to Comply with the reply.

Ron Schwadron, Ph.D.
Primary Examiner
Art Unit 1644

RONALD D. SCHWADRON
PRIMARY EXAMINER
GROUP 1800 1644

Notice to Comply	Application No. 09/982849	Applicant(s) Reff	
	Examiner Ron Schwadron, Ph.D.	Art Unit 1644	

NOTICE TO COMPLY WITH REQUIREMENTS FOR PATENT APPLICATIONS CONTAINING NUCLEOTIDE SEQUENCE AND/OR AMINO ACID SEQUENCE DISCLOSURES

Applicant must file the items indicated below within the time period set the Office action to which the Notice is attached to avoid abandonment under 35 U.S.C. § 133 (extensions of time may be obtained under the provisions of 37 CFR 1.136(a)).

The nucleotide and/or amino acid sequence disclosure contained in this application does not comply with the requirements for such a disclosure as set forth in 37 C.F.R. 1.821 - 1.825 for the following reason(s):

☒ 1. This application clearly fails to comply with the requirements of 37 C.F.R. 1.821-1.825. Applicant's attention is directed to the final rulemaking notice published at 55 FR 18230 (May 1, 1990), and 1114 OG 29 (May 15, 1990). If the effective filing date is on or after July 1, 1998, see the final rulemaking notice published at 63 FR 29620 (June 1, 1998) and 1211 OG 82 (June 23, 1998).

☒ 2. This application does not contain, as a separate part of the disclosure on paper copy, a "Sequence Listing" as required by 37 C.F.R. 1.821(c).

☒ 3. A copy of the "Sequence Listing" in computer readable form has not been submitted as required by 37 C.F.R. 1.821(e).

☐ 4. A copy of the "Sequence Listing" in computer readable form has been submitted. However, the content of the computer readable form does not comply with the requirements of 37 C.F.R. 1.822 and/or 1.823, as indicated on the attached copy of the marked -up "Raw Sequence Listing."

☐ 5. The computer readable form that has been filed with this application has been found to be damaged and/or unreadable as indicated on the attached CRF Diskette Problem Report. A Substitute computer readable form must be submitted as required by 37 C.F.R. 1.825(d).

☐ 6. The paper copy of the "Sequence Listing" is not the same as the computer readable from of the "Sequence Listing" as required by 37 C.F.R. 1.821(e).

☐ 7. Other: see enclosed communication

Applicant Must Provide:

☒ An initial or substitute computer readable form (CRF) copy of the "Sequence Listing".

☒ An initial or substitute paper copy of the "Sequence Listing", as well as an amendment directing its entry into the specification.

☒ A statement that the content of the paper and computer readable copies are the same and, where applicable, include no new matter, as required by 37 C.F.R. 1.821(e) or 1.821(f) or 1.821(g) or 1.825(b) or 1.825(d).

For questions regarding compliance to these requirements, please contact:

For Rules Interpretation, call (703) 308-4216 or (703) 308-2923
For CRF Submission Help, call (703) 308-4212 or 308-2923
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U.S. Patent Appl. No. 09/928,849
Attorney Docket No. 037003-0280728

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re PATENT APPLICATION OF

Mitchell E. Reff

Application No. 09/635,929

Filed: October 22, 2001

Group Art Unit: 1644

Examiner: Ronald Schwadron

Title: VARIANT IgG3 RITUXAN AND THERAPEUTIC USES THEREOF

* * * * *

RESPONSE TO NOTICE TO COMPLY WITH REQUIREMENTS FOR PATENT APPLICATIONS CONTAINING NUCLEOTIDE AND/OR AMINO ACID SEQUENCE DISCLOSURES

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P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

This is in response to the official communication dated October 6, 2004. This response is timely filed.

REMARKS

The official communication states that the instant application contains sequence disclosures that are encompassed by the definitions for nucleotide and/or amino acid sequences set forth in 37 C.F.R. § 1.821(a)(1) and (a)(2). The application is deemed deficient in failing to comply with the requirements of 37 C.F.R. §§ 1.821-1.825 for failing to submit a sequence listing for the sequence "PSAG" disclosed on page 25 of the specification.

Applicants respond that the abbreviation "PSAG" sequence is incorrectly identified as a sequence disclosure. The text of the originally filed application which contains the alleged sequence disclosure is repeated below:

> Alternatively, the anbitody is conjugated to one or more calichearnicin molecules. The calicheamicin family of antibiotics are capable of producing double-stranded DNA breaks at sub-picomolar concentrations. Structural analogues of calicheamicin which may be used include, but are not limited to, γ_1^I, α_2^I, α_3^I, N-acetyl-γ_1^I, PSAG and θ^I_1 (Hinman *et al. Cancer Research* 53: 3336-3342 (1993) and Lode *et al. Cancer Research* 58: 2925-2928 (1998)).

From the referenced paragraph, it is clear that PSAG is a structural analogue of the antibiotic calicheamicin, not a nucleotide or amino acid sequence within the meaning of 37 C.F.R. § 1.821(a)(1) and (a)(2). The Hinman et al. journal article (copy enclosed), which is cited in the same sentence as PSAG, fully describes the PSAG calicheamicin structural analogue. *See e.g.*, page 3337, col. 2, paragraph 2, and Figure 1. Specifically, PSAG is a pseudoaglycone calicheamicin derivative. Based thereon, it is respectfully requested that the requirements for filing of a sequence listing according to 37 C.F.R. §§ 1.821-1.825 be withdrawn.

CONCLUSION

If any points regarding the sequence listing remain in issue, which the examiner feels may be best resolved through a personal or telephone interview, he is kindly requested to contact the undersigned attorney at the telephone number listed below.

Respectfully submitted,
PILLSBURY WINTHROP LLP

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Date: October 26, 2004

TAC/JBM

[CANCER RESEARCH 53, 3336-3342, July 15, 1993]

Preparation and Characterization of Monoclonal Antibody Conjugates of the Calicheamicins: A Novel and Potent Family of Antitumor Antibiotics

Lois M. Hinman,[1] Philip R. Hamann, Roslyn Wallace, Ana T. Menendez, Frederick E. Durr, and Janis Upeslacis

Medical Research Division, American Cyanamid Company, Lederle Laboratories, Pearl River, New York 10965

ABSTRACT

The calicheamicin family of antitumor antibiotics are capable of producing double-stranded DNA breaks at sub-picomolar concentrations. Their potency suggested that the calicheamicins would be excellent candidates for targeted delivery and a hydrazide prepared from the most potent and abundant of the naturally occurring derivative, γ_{1I}, was linked to oxidized sugars on CT-M-01, an internalizing anti-polyepithelial mucin antibody. The conjugates retained the immunoreactivity of the unmodified antibody and were specifically cytotoxic toward antigen positive tumor cells *in vitro* and *in vivo*. Hydrazide analogues of less potent calicheamicin derivatives were also prepared and conjugated to CT-M-01. Comparison of the therapeutic efficacy of the conjugates against the MX-1 xenograft tumor implanted s.c. in nude mice showed that conjugates of derivatives missing the rhamnose, a sugar residue that is part of the DNA binding region of the drug, were not as promising as antitumor therapies. However, conjugates of two derivatives, α_{3I} and *N*-acetyl-γ_{1I}, in which the rhamnose residue is present but the amino sugar residue of the parent drug is either missing or modified, significantly inhibited tumor growth over a 4-fold dose range and produced long-term tumor-free survivors. Sterically hindering methyl groups adjacent to the disulfide in the linker further increased the therapeutic window of these potent conjugates.

INTRODUCTION

Over the past decade, many MoAb[2] conjugates of radioisotopes, protein toxins, and cytotoxic drugs have been prepared and tested in model systems. Radiolabeled MoAbs have proven to be effective imaging agents in the clinic and are showing some promising therapeutic results for the treatment of lymphomas and leukemias (1–4). Protein toxin-MoAb conjugates (immunotoxins) are likewise progressing in clinical trials, particularly for the treatment of lymphomas (5). In contrast, clinical progress with cytotoxic drug-MoAb conjugates has been less promising, despite encouraging preclinical data (6–8). An important factor limiting the success of drug-MoAb conjugates is the relatively low potency of standard chemotherapeutics, which is further reduced by their conjugation to MoAbs (9). With most clinically used anticancer drugs, a large number of drug molecules must be taken up by each cell to achieve cell death. Poor tumor penetration, low antigen expression, and antigenic heterogeneity limit the number of MoAb-targeted drug molecules that can reach each cell (10), and with many standard drugs, that number is too low to produce clinically significant antitumor effects. In contrast, the protein toxins such as ricin, which kill cells in a catalytic manner, have been used to prepare immunotoxins (protein-MoAb conjugates) that have produced highly significant antitumor effects *in vivo*. However, the inherent immunogenicity of the protein toxins themselves compromises the clinical utility of immunotoxins in all except severely immunocompromised patients (11–13).

Recently, several groups have concentrated their targeting research on low molecular weight cytotoxics with potencies intermediate between the protein toxins and anticancer agents such as vincristine or Adriamycin. Preclinical evaluations of conjugates of highly potent low molecular weight cytotoxics such as the trichothecenes (14) and maytansines (15), both of which are significantly more potent than vincristine, have been reported. In our own work, we have used a new family of particularly potent anti-tumor antibiotics, the calicheamicins, which were originally identified by their impressive potency in a screen for DNA damaging agents (16). The antibiotics, isolated from a broth extract of a soil microorganism *Micromonospora echinospora calichensis*, were termed the calicheamicins and later were identified as members of a new class of potent enediyne containing antibiotics which includes the esperamicins, dynemycin, and neocarzinostatin (17). The binding of the most potent of the calicheamicins, γ_{1I}, in the minor groove of DNA and the resulting sequence-specific DNA cleavage has been described (18, 19). The calicheamicins are relatively small molecules with molecular weights in the range of M_r 1500. The small size combined with a unique mechanism of action and extreme potency suggested that the calicheamicins might be good candidates for MoAb targeting.

In this article, we describe the structure-activity profile of a series of calicheamicin analogues conjugated to the anti-PEM MoAb, CT-M-01, which binds to an internalizing antigen present on a number of solid tumor types including breast, ovarian, colon, and non-small cell lung carcinomas. The goals of these studies were 3-fold: (*a*) to determine the feasibility of preparing calicheamicin conjugates with antitumor activity; (*b*) to investigate the correlation between the potency of structural analogues of the calicheamicins and their therapeutic potential as conjugates; and (*c*) to test linker variations in an effort to optimize the therapeutic window for the conjugates.

MATERIALS AND METHODS

Monoclonal Antibodies. CT-M-01, also known as 7F11C7, is an internalizing IgG_1 that recognizes the PEM antigen, located preferentially on the cell surface of human cancerous epithelial cells. Originally developed to recognize the human milk fat globule membrane of breast carcinomas, CT-M-01 binds with high affinity to a broad spectrum of solid tumors and is internalized into its target cells after binding (20). The murine CT-M-01 and MOPC-21 used in these studies were produced and purified from tissue culture supernatants by Celltech, Ltd., United Kingdom. MOPC-21, a secreted murine myeloma IgG_1 that does not bind to any mouse antigen or human xenograft tumors, was used to prepare nonbinding isotype-matched MoAb conjugates (21, 22). Lym 2, an IgG_1 reactive with human B-lymphocytes, but not with solid tumor lines, was used as a negative control in the *in vitro* binding studies (23) and was produced and purified from tissue culture supernatants by Techniclone, Inc.

Iodination of MoAbs for use in the competitive binding assays was accomplished using the diiodinated Bolton Hunter reagent, *N*-succinimidyl 3-(4-hydroxy-3-[^{125}I]diiodophenyl)propionate, purchased from New England Nuclear. The MoAb was iodinated at pH 7.4 in phosphate buffer at a protein concentration of 1.25 mg/ml, using 5 mCi of Bolton-Hunter reagent for each 2.5 mg of protein iodinated. The labeled MoAb was purified by gel filtration chromatography and exhaustive dialysis. The specific activity of the ^{125}I-CT-M-01 ranged from 0.3 to 0.5 μCi/μg protein and the labeling did not interfere with the antigen binding of the MoAb.

Cells and Culture Methods. An MX-1 human breast carcinoma cell line, established in this laboratory, was used as the experimental target for CT-M-01

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[1] To whom requests for reprints should be addressed.

[2] The abbreviations used are: MoAb, monoclonal antibody; PEM, polyepithelial mucin; PBS, phosphate buffered saline; MTD, maximum tolerated dose; MED, minimum effective dose; TR, therapeutic ratio (= MTD/MED); DMF, dimethyl formamide; OD, optimal dose; DM, dimethyl; PSAG, pseudoaglycone; γ_{1I}-DM-hyd, γ_{1I} dimethyl hydrazone; γ_{1I}-hyd, γ_{1I} hydrazone; t, triplet.

in vitro (24). The tissue culture line was derived as a clonal isolate in agarose medium from a tumor transplant of human breast carcinoma (MX-1) growing in athymic mice. MX-1 cells were propagated in RPMI 1640 containing 5% fetal calf serum, 5 μg/ml insulin and transferrin, 5 ng/ml selenium, and 50 μg/ml gentamicin. Cultures were maintained in a humidified, 5% CO_2 incubator at 36°C and were subcultured once each week by scraping the loosely attached cells from the culture flasks. The MX-1 cells bind approximately 200,000 molecules of CT-M-01/cell *in vitro*, approximately 25% of which is internalized within 4 h.[3] The XC rat sarcoma cell line was obtained from the Naval Biomedical Laboratory, Oakland, CA. These cells were propagated as monolayers and subcultured following dispersal with 0.25% trypsin. For *in vitro* cytotoxicity tests, streptomycin, 50 μg/ml and penicillin, 50 units/ml, were incorporated into the medium.

Immunoreactivity of the MoAbs and conjugates was measured by a direct radioimmunoassay comparing the competitive binding of the test sample with that of iodinated CT-M-01. For each assay, 10^6 MX-1 cells in 0.1 ml were incubated with 0.05 ml of 4 μg/ml ^{125}I-CT-M-01 (specific activity, ~0.3–0.5 μCi/μg) and 0.05 ml of serial 4-fold dilutions of the test samples, the highest concentration being 200 μg/ml. After a 1-h incubation, the cells were washed 3 times with Dulbecco's PBS and transferred to fresh tubes and counted. Binding inhibition curves were plotted and 50% inhibitory concentration values for each conjugate were compared with that of the unmodified control antibody as a relative measure of retention of immunoreactivity.

***In Vitro* Cytotoxicity Assays.** To evaluate cytotoxicity in drug or conjugate samples, viable cells (10^5/0.2 ml) were aliquoted into 15-ml test tubes which contained 0.2 ml of the sample to be tested at the appropriate concentration. Concentrations were all normalized to microgram equivalents of calicheamicin γ_{1}^{I}. Tubes were vortexed and incubated at 37°C for 7 min, and the pellets washed 3 times with 8 ml of medium. One ml of medium was added to each pellet, the cells were vortexed, and 0.2 ml was removed and placed in a well of a 96-well plate (2×10^4 cells). These cells were incubated for 3 days, at which time 0.1 ml of supernatant was removed and replaced with 0.2 mCi of [^{3}H]thymidine in 0.1 ml of fresh medium. Incubation was resumed for an additional 24 h at which point the cells were harvested and counted. Growth inhibition curves of each drug or conjugate were plotted and the 50% inhibitory concentration value (concentration of drug equivalents needed for 50% [^{3}H]-thymidine uptake inhibition) of each sample was determined.

***In Vivo* Tests for Anti-tumor Activity.** Drug and drug hydrazides were tested for antitumor activity against lymphocytic leukemia P388 in mice according to the protocol described by Geran *et al.* (25). As expected, the MoAb conjugates which do not recognize the murine tumors were inactive in this system and were evaluated instead for antitumor effects against human breast xenograft tumors implanted in athymic mice by procedures previously described (26). The two breast carcinomas studied were the ductal cell MX-1 and undifferentiated MX-2, both obtained as xenograft transplants from the Division of Cancer Treatment and the Division of Cancer Prevention of the National Cancer Institute. Tumors were implanted s.c. into athymic mice and test samples were inoculated i.p. or i.v. at several dose levels, every 4 days for a total of 3 doses, starting 2–3 days after tumor implantation. Each test group contained 6 mice and in each test a control group of 10 mice were given injections of a volume of PBS, pH 7.4 (the conjugate vehicle), equivalent to the volume of the highest conjugate dose (usually 0.5 ml). Tumor mass was determined by measuring the tumor diameter once weekly for 35–49 days post-tumor implantation. Significant antitumor activity was defined as a sustained 58% inhibition of mean tumor mass compared with untreated controls in groups with greater than 65% survivors. A TR was defined as the MTD/MED and used as a measure of the therapeutic window for the conjugates or drugs tested.

Preparation of Thiol Hydrazides. 3-Mercaptopropionyl hydrazide used in the preparation of the "simple" hydrazide conjugates was prepared as follows: 9.2 ml of methyl 3-mercaptopropionate (83 mmol) were added dropwise over 2 h to 5.4 ml (3 eq) of anhydrous hydrazine in 100 ml of refluxing tetrahydrofuran under argon. After an additional 2 h at reflux, the reaction mixture was cooled and the solvent was removed in a vacuum. The excess hydrazine was removed by addition of toluene and reconcentration. The crude product was purified by flash silica gel chromatography eluting first with 5% ethyl acetate in chloroform and then 20% methanol in chloroform.

3-Mercapto-3-methylbutyryl hydrazide used in the preparation of the "dimethyl" hydrazides was prepared as follows: 9 ml (1.3 eq) of thiolacetic acid was added to 10 g of 3,3-dimethyl acrylic acid. This mixture was heated at reflux under argon for 6 h. The excess thiolacetic acid was removed under aspirator vacuum and the resultant oil was dissolved in 100 ml absolute ethanol containing 200 μl of concentrated sulfuric acid. This reaction was refluxed for 24 h before adding 16 ml of hydrazine and then for an additional 24 h under argon. The reaction mixture was concentrated and the residue was dissolved in a mixture of brine and saturated sodium bicarbonate. The product was extracted with several volumes of chloroform. The combined chloroform layers were dried with magnesium sulfate, filtered, and reduced in volume to an oil. This oil was purified by flash chromatography on silica gel with a methanol-chloroform gradient and then crystallized from chloroform-hexane to give 3-mercapto-3-methylbutyryl hydrazide as a low-melting solid.

Calicheamicin Analogues. The γ_{1}^{I}, α_{2}^{I}, and α_{3}^{I} calicheamicins were all isolated from the fermentation broth of *M. echinospora calichensis* as described previously (27). The structural elucidation of these compounds, the isolation of the pseudoaglycone derivative, and the synthesis of *N*-acetyl calicheamicin γ_{1}^{I} from calicheamicin γ_{1}^{I} have been described in detail as well (28).

Hydrazide derivatives of the calicheamicins were prepared by displacement at the methyltrisulfide moiety of the analogues with the mercaptohydrazides described above. Preparation of the calicheamicin γ_{1}^{I} hydrazide typifies this procedure: 70 mg (0.051 mmol) of calicheamicin γ_{1}^{I} in 100 ml of acetonitrile at −15°C was added to 13.2 mg (2 eq) of 3-mercaptopropionyl hydrazide in 1 ml of acetonitrile. The reaction was warmed to 4°C for 24 h, and then the solvent was removed *in vacuo*. The crude product was purified by flash chromatography on Merck silica gel (packed with carbon tetrachloride) using a gradient of 5–15% methanol in chloroform to give 55 mg of a yellowish glass. This material can be used directly or dissolved into chloroform containing a trace of methanol and precipitated by being poured into a rapidly stirring 1:1 mixture of ether-hexane to give a white powder which can be stored indefinitely at −15°C. The high-performance liquid chromatography retention time for this compound is 5.0 min with 41% acetonitrile/0.1 M aqueous NH_4OAc (Zorbax ODS C-18, 4.6 mm x 25 cm column; 2 ml/min) (for calicheamicin γ_{1}^{I} retention time is 5.5 min with 56% acetonitrile). Mass spectrum (FAB) is 1408 ($M + H^+$), 1430 ($M + Na^+$); and with added acetone, 1448 ($M + 40 + H^+$, acetone hydrazone). The nuclear magnetic resonance (300 mHz, $CD_3OD/CDCl_3$) of this compound is virtually identical to the published spectrum for calicheamicin γ_{1}^{I} (26) except for the following: absence of a CH_3-methyl trisulfide resonance and a new resonance at 2.97 ppm (2H, t, $J_{a,b}$ = 7Hz, CH_2), 2.56 (2H, t, $J_{a,b}$ = 7Hz, CH_2).

Conjugation of Calicheamicin Hydrazides to MoAbs. The method used for the preparation of MoAb conjugates involved reactions with carbohydrate-derived aldehydes, as previously described (29). The conjugation of all the hydrazide and dimethyl hydrazide derivatives followed a similar procedure as outlined below. MoAb, at a concentration of 5 mg/ml in 50 mM sodium acetate buffer, pH 5.5, was oxidized at 4°C for 30 min with 12.5 mM sodium periodate (prepared freshly as a 100 mM stock in the acetate buffer). The oxidized antibody was desalted by gel filtration (PD-10 columns) and then reacted at 25°C with a 30-fold molar excess of calicheamicin γ_{1}^{I} hydrazide in DMF as described below. A typical conjugation reaction mixture contained approximately 3 mg/ml protein and 1 mg/ml drug hydrazide in a mixed solvent consisting of 50 mM sodium acetate buffer, pH 5.5, containing 15% DMF. After 3 h, the reaction was quenched with 5% of the reaction volume of 0.2 M $NaCNBH_3$ and 1% (v/v) of the reaction volume of 1 M acethydrazide for 1 h. The $NaCNBH_3$ reduction step was included in all the experiments in this article. However, subsequent studies indicated under the conditions used in this study little, if any, reduction of the hydrazone moiety occurred and conjugates made with and without reduction showed no differences, including in biological properties. Acethydrazide does function in blocking unreacted free aldehyde groups and minimizing aggregation and therefore is routinely included in all aldehyde-based conjugation reactions. To terminate the conjugation reactions, conjugates were passed through a desalting column equilibrated with 50 mM phosphate buffer, 100 mM NaCl, at pH 6.5, and exhaustively dialyzed against the same buffer. Monomeric conjugates were separated from dimers and higher order aggregates by S-200 gel exclusion chromatography.

The drug concentrations of calicheamicin derivatives and drug loading on conjugates were determined spectroscopically. The extinction coefficient de-

[3] C. P. Beyer, unpublished results.

termined for a calicheamicin γ_1^I β-mercaptopropionic acid disulfide of 4010 M^{-1} at 333 nm (in 10% DMF in PBS), a species that represents the attached disulfide form of the drug, was used as a standard. Since none of the structural modifications of the calicheamicins described here significantly affect the chromophore of the drug, this extinction coefficient was adjusted for variations in the molecular weight of the analogues and used for all studies. The molecular weights for the hydrazide calicheamicin derivatives included in this study are: 1408 for γ_1^I hydrazide, 1248 for α_2^I hydrazide, 1091 for pseudoglycone hydrazide, 1251 for α_3^I hydrazide, 1450 for *N*-acetyl-γ_1^I hydrazide, 1436 for γ_1^I dimethyl hydrazide, and 1478 for *N*-acetyl-γ_1^I dimethyl hydrazide. Thus, for example, for γ_1^I hydrazide conjugates, drug concentration (μg/ml) = A_{333}/ϵ_{333} (ml/mg) using ϵ_{333} (ml/mg) = 2.85 (4010/1408). The contribution to protein absorbance made by the calicheamicin at 280 nm was estimated to be 3 times the absorbance value calculated for the drug at 333 nm. Using ε 333 (ml/mg) = 1.43 as the standard extinction coefficient for an IgG molecule, a corrected antibody concentration (mg/ml) was calculated as:

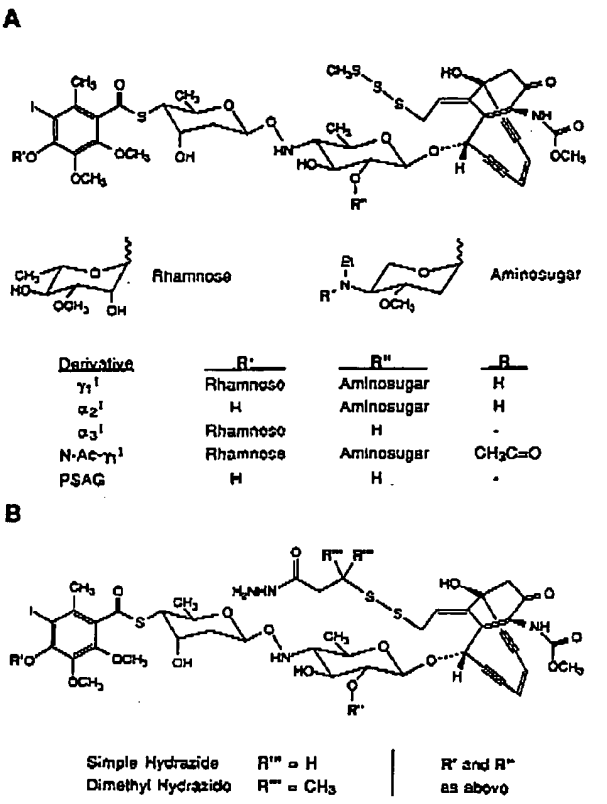
$$\frac{A_{280} - (3 \times A_{333})}{1.43}$$

These spectroscopic values proved convenient for routinely measuring drug and MoAb concentrations in the conjugates and were confirmed using radiolabeled drug and independent determinations of protein concentration using standard BioRad reagents and assay procedures.

RESULTS

Characterization of Calicheamicin Derivatives for Conjugation. The five structural analogues of calicheamicin used in this study have been previously designated γ_1^I, α_2^I, α_3^I, *N*-acetyl-γ_1^I, and PSAG. The structures of these derivatives, described in detail elsewhere (26, 27), are shown in Fig. 1*A*. The core of the molecule shown in Fig. 1*A*, which includes the methyl trisulfide "trigger" which undergoes reduction to cause a molecular rearrangement of the enediyne bicyclic "warhead" (the part of the molecule that generates a diradical that produces double-strand DNA breaks) and the sugar/aromatic ring "backbone" is common to all five of the analogues used in these studies. Structural variations relate to the presence or absence of the rhamnose (at R') and/or aminosugar (at R'') as indicated. The most potent "parent" compound, calicheamicin γ_1^I, contains both the rhamnose and the aminosugar. The α_2^I analogue is missing the rhamnose, while α_3^I is missing the aminosugar and PSAG lacks both the rhamnose and aminosugars. The fifth analogue, *N*-acetyl-γ_1^I, was prepared by acetylation of the amino sugar of γ_1^I (27). The two hydrazides ("simple" and "dimethyl") used in these studies are shown in Fig. 1*B*. It should be noted that the hydrazides are disulfide versions of the trisulfide "parent" compounds and were prepared from the trisulfide analogues for conjugation to MoAbs containing periodate oxidized sugars.

The anti-tumor effects of the five parent calicheamicin derivatives, along with their respective hydrazides, were compared *in vivo* in the P388 leukemia model (Table 1). In each experiment, the test drug was administered i.p. to normal mice carrying P388 leukemia and a comparable group of nontumored animals. The lethality of the drug in the nontumored animals was used to determine MTD. In Table 1, we report the percentage of increase in life span for each derivative at two doses: the OD that gives the greatest percentage of increase in life span in the P388 animals, and the MTD, determined in the nontumored animals. Several conclusions can be made from these data. For all five derivatives, the MTD was less than the OD. For example, the greatest increase in life span resulting from treatment with any of these derivatives was 150% for the α_2^I calicheamicin, at a dose 8-fold higher than the MTD. The potency of the hydrazides was 2–8-fold less than that of the corresponding parent compounds for all analogues. From these data it is clear that although the calicheamicins are highly potent, toxicity limits their therapeutic efficacy as single agents



Derivative	R'	R''	R
γ_1^I	Rhamnose	Aminosugar	H
α_2^I	H	Aminosugar	H
α_3^I	Rhamnose	H	-
N-Ac-γ_1^I	Rhamnose	Aminosugar	$CH_2C{=}O$
PSAG	H	H	-

Fig. 1. In *A*, structural analogues of the calicheamicins which vary in the integrity of the rhamnose and amino sugars are outline schematically. *B*, comparable hydrazide analogues prepared from each derivative are shown.

Table 1 *Comparison of antitumor effects and lethality of calicheamicin analogues and hydrazides*

	% of increase in life span[a]			
	Parent drug		Hydrazide	
Derivative	OD (μg/kg)	MTD (μg/kg)	OD (μg/kg)	MTD (μg/kg)
γ_1^I	5 (123)	1.25 (86)	5 (83)	2.5 (75)
α_2^I	10 (150)	1.25 (75)	10 (130)	5 (83)
α_3^I	40 (109)	10 (73)	80 (73)	80 (73)
N-Acetyl-γ_1^I	40 (123)	20 (79)	160 (63)	160 (63)
PSAG	160 (73)	40 (60)	Not tested	

[a] Numbers in parentheses, percentages.

against P388 leukemia. Similar dose-limiting toxicities also were seen for these calicheamicin analogues when they were studied as single agents in other murine tumors, such as B16 melanoma (30), and in the xenograft tumors as well (see below). To test the potential of these compounds as targeted agents, monomeric conjugates were prepared from each of the five hydrazide analogues. These hydrazone conjugates had drug loadings of 2 to 3 molecules of calicheamicin equivalents/MoAb molecule and retained greater than 85% of the immunoaffinity of the unmodified MoAb.

Activity and Specificity of CT-M-01-γ_1^I Hydrazone Conjugates. Calicheamicin γ_1^I, the calichamicin γ_1^I hydrazide, and two conjugates, a CT-M-01-γ_1^I hydrazide conjugate that binds to the MX-1 cells and a nonbinding control Lym-2-γ_1^I hydrazide conjugate, were com-

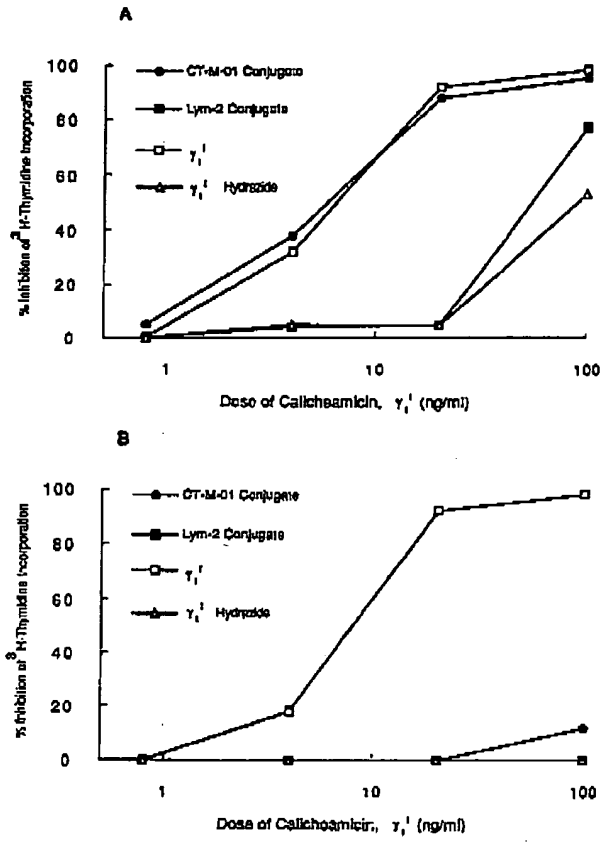


Fig. 2. A comparison of the *in vitro* cytotoxicity and specificity of γ_{1I}, γ_{1I} hydrazide, and conjugates of γ_{1I} hydrazide with CT-M-01 and Lym-2 on the MX-1 (*A*) and XC-RM cells (*B*). Cells at a concentration of 10^5 cells/ml were incubated for 7 min with 3-fold dilutions of γ_{1I}, γ_{1I} hydrazide, or γ_{1I} hydrazide conjugates over a dose range of 0.8 to 100 ng/ml. After 3 days of growth, the cells were pulsed with [^{3}H]thymidine and the inhibition of cell growth resulting from the treatment was calculated relative to the growth of the untreated control cells.

pared for their ability to inhibit the growth of two cells lines *in vitro*. In Fig. 2 the cytotoxic effects of five-fold dilutions of calicheamicin γ_{1I}, γ_{1I} hydrazide, or γ_{1I} hydrazide conjugates with these two antibodies were compared on the MX-1 breast carcinoma cells, which express the PEM antigen and binds and internalizes CT-M-01 (Fig. 2*A*) and the rat sarcoma XC cells, which do not bind either antibody (Fig. 2*B*). A comparison of the titration data for the conjugates and drugs on these cell lines show that the CT-M-01 conjugate and γ_{1I} were equivalently cytotoxic to the MX-1 cells, at a 50% inhibitory dose of approximately 8 ng/ml, while only the parent drug itself showed this level of activity toward the nontarget line. The nonbinding Lym-2 conjugate and γ_{1I} hydrazide itself are at least 10-fold less active than the CT-M-01 conjugate or γ_{1I} on the MX-1 line, although this Lym 2 conjugate was highly active against its own antigen-positive target line, HS Sultan, under similar experimental conditions (data not shown).

Antitumor effects of the CT-M-01-γ_{1I} hydrazide conjugate were studied *in vivo* against the MX-1 human breast carcinoma implanted s.c. in nude mice. The conjugate was administered at 3 dose levels (as 3 doses given 4 days apart). The top dose (25 μg/kg) was lethal to all animals treated by day 21 (data not shown), while at lower doses the conjugates produced a dose related inhibition of tumor growth (Fig. 3). The lowest dose tested (6.3 μg/kg × 3) was nonlethal but minimally effective and a dose of 12.5 μg/kg × 3 produced a significant reduction in the size of the xenograft tumor from 21 to 35 days post-tumor implantation, with 4 out of 6 of the test animals in that dose group surviving at day 35. In this study, calicheamicin γ_{1I} itself was lethal at one-tenth the MTD for the conjugates (MTD < 2.5 μg/kg) and the drug hydrazide and noncovalent mixture of the drug hydrazide and MoAb (prepared to simulate the concentrations of both drug and MoAb in the conjugates) were both lethal at <6.3 μg/kg. The γ_{1I} and γ_{1I} hydrazide were not effective at nontoxic doses and CT-M-01 itself did not inhibit the growth of the tumor at doses up to 0.5 mg/dose × 3 doses (>10 times the dose administered in this test).

From these results it was clear that conjugation of calicheamicin γ_{1I} to CT-M-01 increased the MTD of calicheamicin γ_{1I} more than 10-fold and, more importantly, allowed the delivery of a therapeutically effective dose of drug to the tumor. However, treatment with the CT-M-01-γ_{1I} hydrazone conjugates did not produce any complete regressions of the MX-1 tumor or result in any long-term survivors. Based on activity against the MX-1 tumor *in vivo*, as presented in Fig. 3 and summarized in Table 2, a TR of ~1 could be estimated for CT-M-01-γ_{1I} hydrazone conjugates which compares favorably with the fractional TRs of the drug derivatives or drug-MoAb mixtures. In an effort to further increase the TR for these conjugates, conjugates of other calicheamicin derivatives were also evaluated.

***In Vivo* Activity of CT-M-01 Conjugates of Calicheamicin Variants.** Hydrazide derivatives of the four other calicheamicins described above were conjugated to CT-M-01 and tested *in vivo* against the MX-1 tumor. As summarized in Table 2, structural variations in the drug had a profound effect on the therapeutic efficacy of their conjugates which did not necessarily correspond with the activity of the drugs as single agents (Table 1). For example, the CT-M-01 conjugate of α_{2I}, the analogue missing the rhamnose from the putative DNA binding region of the drug and next in potency to γ_{1I} against P388, had a TR <1 and showed no antitumor effects at nonlethal doses (Fig. 4) and the conjugates were inactive on antigen-negative xenograft tumors.

In contrast, conjugates of α_3 and *N*-acetyl-γ_{1I}, analogues which contain the rhamnose but are modified at the amino sugar, were highly efficacious over a 4-fold dose range, showing an improved therapeutic window compared with γ_{1I} and a TR >4 (Table 2). As shown in Fig. 5, *A* and *B*, both of these conjugates had dramatic antitumor activity, inhibiting the growth of the MX-1 tumor at nonlethal doses and producing long-term tumor-free survivors (>100 days) at the 100-μg/kg dose. The parent drug derivatives included in the tests as controls were inactive at nonlethal doses.

The fifth variant of calicheamicin conjugated to CT-M-01 was PSAG, missing both the rhamnose and the amino sugar. PSAG is significantly less potent than the other derivatives against P388 leukemia. As shown in Table 2, this conjugate was ineffective and nonlethal even at doses 28-fold higher than the MTD for the γ_{1I} conjugate, indicating that the delivery of the "warhead" of calicheamicin on a MoAb was not sufficient to retain activity. Because of this low potency, further investigations of PSAG conjugates were not pursued.

Effects of Variations in the Hydrazone Linkage on *in Vivo* Activity. Changes in the linker region of the calicheamicin hydrazide conjugates were also examined as a means of increasing the TR of the calicheamicin conjugates. Methyl groups that add steric bulk adjacent to the disulfide were introduced into the hydrazide linker as shown in Fig. 1*B*. A comparison of the antitumor activity of a "simple" γ_{1I} hydrazone (γ_{1I}-hyd) and a γ_{1I}-DM-hyd conjugate was made on the MX-1 tumor *in vivo*. As summarized in Table 2, the γ_{1I}-DM-hyd conjugate had strong antitumor activity at 6 μg/kg, a dose which is ineffective with the simple γ_{1I}-hyd conjugate (Fig. 3). In addition, no lethality was seen with the γ_{1I}-DM-hyd conjugate at the 12.5 μg/kg

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Table 2 *Comparison of efficacy of calicheamicin-CT-M-01 hydrazide conjugates*

Derivative	Sugar: Rhamnose	Sugar: Amino	Growth of the MX-1 tumor in athymic mice: MED[a] (μg/kg × 3)	MTD[a] (μg/kg × 3)	TR MTD/MED	Tumor size[a] (% of Control)	Tumor-free survivors at day 100
γ_1^I-hyd	+	+	12.5	12.5	1	13.6	0 of 6
α_2^I-hyd	−	+	50.0	30.0	<1	43.6	0 of 6
α_3^I-hyd	+	−	<25.0	>100.0	>4	0.0	6 of 6
N-Ac-γ_1^I-hyd	+	Modified	<25.0	>100.0	>4	0.0	6 of 6
PSAG-hyd	−	−	>350.0	>350.0	<1	78.7	0 of 6
γ_1^I-DM-hyd	+	+	6.0	>12.5	2	0.0	6 of 6[b]
N-Ac-γ_1^I-DM-hyd	+	+	<50.0	300.0	>6	0.0	6 of 6
MOPC-21 (nonspecific) *N*-Ac-γ_1^I-DM-hyd	+	+	>300.0	300.0	<1	63.0[c]	

[a] Measured at 35 days post-tumor implantation; $n = 6$ per test group; $n = 10$ in the control group.
[b] Tumor-free survivors for >49 days, at which time the animals were sacrificed.
[c] Measured on day 28, no tumor-free animals on day 28.

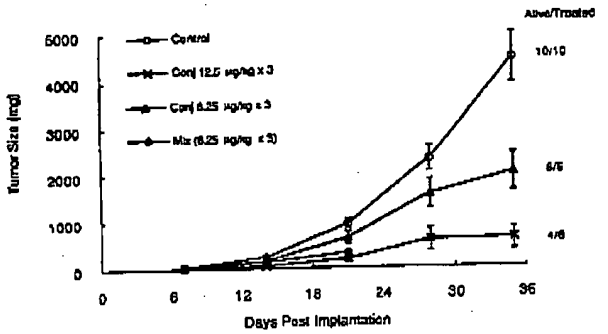

Fig. 3. Antitumor effects of a CT-M-01 conjugate of γ_1 hydrazide and controls on the MX-1 xenograft tumor. Athymic mice implanted with the s.c. MX-1 breast carcinoma were treated QD4 × 3, i.p. with the conjugate and appropriate controls over a range of doses from 6.25 to 25 μg/kg of drug/dose × 3 doses, delivered on a total of 0.6 to 12.5 μg of CT-M-01/animal. The γ_1 itself included in the test was lethal at doses >0.5 μg/kg, the MTD for the γ_1 hydrazide was <6.25 μg/kg. The antitumor response of a CT-M-01 mixture with γ_1 hydrazide is shown. The conjugate was lethal at 25 μg/kg (data not included) and produced a significant antitumor response at the 12.5 μg/kg dose. *Right*, the number of animals alive in each treatment group at day 35. In all test groups, $n = 6$; in the control group, $n = 10$; *bars*, ± SEM for each data point.

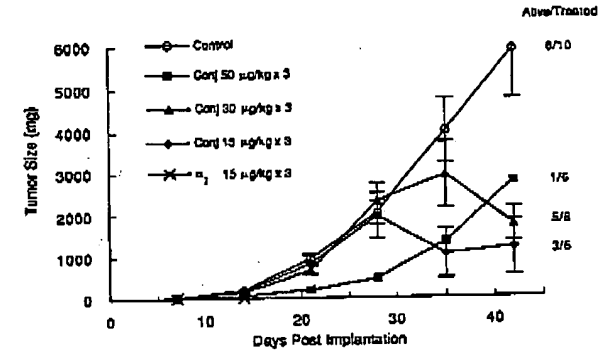

Fig. 4. Antitumor effects of a CT-M-01 conjugate of α_{2I} hydrazide and controls on the MX-1 xenograft tumor. Athymic mice implanted with the s.c. MX-1 breast carcinoma were treated Q4D × 3, i.p. with the conjugate, and drug and antibody controls over a range of doses from 15 to 50 μg/kg of drug/dose × 3 doses. The α_{2I} itself was included in the test and was lethal at a dose of 15 μg/kg × 3. The only dose of conjugate that showed strong antitumor effects in this study was 50 μg/kg, which was lethal at 42 days. *Right*, the number of animals alive in each treatment group at day 42. In all test groups, $n = 6$; and in the control group, $n = 10$; *bars*, ± SEM for each data point.

dose, while with the simple γ_1-hyd conjugate, 2 of the 6 animals at that dose were dead at day 35. In fact, with the γ_1-DM-hyd conjugate, all animals treated with the 12.5 μg/kg dose of conjugate were tumor-free at the 49-day observation point. The increased efficacy at low doses and decreased lethality at the higher doses resulted in an increased TR for the γ_1-DM-hyd over the simple γ_1 hyd conjugate.

CT-M-01 conjugates of *N*-acetyl γ_1 and α_3 calicheamicins were also prepared incorporating the hindered dimethyl hydrazone linkages. A comparison of the antitumor activity of *N*-acetyl γ_1 conjugates prepared with the simple and DM-hydrazone linkages, tested on the MX-2 breast carcinoma, is shown in Fig. 6. At both dose levels tested, the DM-hydrazone conjugate was more effective than the simple hydrazone conjugate against this tumor producing 100% tumor-free survivors at both doses. The *N*-acetyl γ_1 and α_3 conjugates, when prepared with the DM hydrazide linkage, have demonstrated significant antitumor effects with TR of four or higher and have produced long-term tumor-free survivors in a number of other antigen-positive tumor models without showing any significant activity on antigen-negative tumors.[4] As shown in Table 2, a CT-M-01 conjugate with *N*-acetyl γ_1-DM-hyd had a TR of >6 against the antigen-positive MX-1 tumor. An equivalent *N*-acetyl γ_1-DM-hyd conjugate prepared with the nonbinding IgG_1, MOPC-21, had a TR of <1 under comparable experimental conditions. As indicated in Table 2, the MOPC-21 conjugate showed only a slight reduction in the tumor mass at MTD (300 μg/kg × 3) and produced no tumor-free survivors. Our studies defining the activity profile of different calicheamicin derivatives conjugated with a variety of tumor-selective, internalizing MoAbs are continuing.

DISCUSSION

In this article we present data that demonstrate the therapeutic potential of MoAb conjugates prepared from a novel class of antitumor antibiotics, the calicheamicins. Despite the dose-limiting toxicities seen with the parent calicheamicins, modifications have been made in both the drug and the linker to produce constructs with a significant therapeutic window. These conjugates are antigen specific and have produced dose-dependent inhibition of tumor growth without lethality in all antigen-positive xenograft tumors tested. The antibody used in these studies was CT-M-01, an internalizing anti-PEM MoAb which binds to a mucin antigen abundant on a number of solid tumors including breast, non-small cell lung, ovarian and colon carcinomas. Our results with CT-M-01 conjugates as well as our previous preliminary reports suggest that internalizing antibodies act as effective surrogate carriers for potent drugs such as the calicheamicins allowing them to bypass the normal mechanisms of nonselective drug uptake, thus rendering them less toxic (28, 31, 32).

Our efforts to optimize the targeting of the calicheamicins involved the evaluation of conjugates prepared from a number of structural analogues of the drug. Using information available on the relative potencies of various calicheamicin analogues in the P388 leukemia

[4] Unpublished results.

model and the MTD of the various analogues in normal mice, we explored structural features of the drug essential for its activity. The calicheamicins bind in the minor groove of DNA and make double-stranded cuts with a specificity for the TCCT/AGGA tetramer (19). We have shown that conjugates of γ_{1}^{I} make equivalent DNA cuts in target cells (28), suggesting that DNA cleavage is the mode of action of the drug, even after conjugation.

Among the four calicheamicin analogues compared with the parent γ_{1}^{I} in this study, two analogues, α_{3}^{I} and PSAG, were missing the rhamnose at the end of the DNA binding region and in neither case were these agents effective as conjugates *in vivo*. Conjugates prepared with α_{3}^{I} and *N*-acetyl-γ_{1}^{I} derivatives, in which the DNA binding region is intact, yet the amino sugar has been eliminated or modified, showed a clear therapeutic advantage over the γ_{1}^{I}, α_{2}^{I}, and PSAG conjugates. Although the function of the amino sugar itself in the calicheamicin structure has not been established, it has been speculated to play a role as a carrier to transport the drug into cells or across membranes. The α_{3}^{I} and *N*-acetyl-γ_{1}^{I} DM hydrazide conjugates may owe their favorable profile to the fact that the DNA binding region of the drug has remained intact while the internalizing antibody can serve as a surrogate for the amino sugar, bringing the potent drug into the target cells. Our data suggest that an intact DNA binding region of calicheamicin is required for optimal antitumor activity, although others have reported the potent antitumor effects of certain enediyne molecules missing a DNA binding region (33).

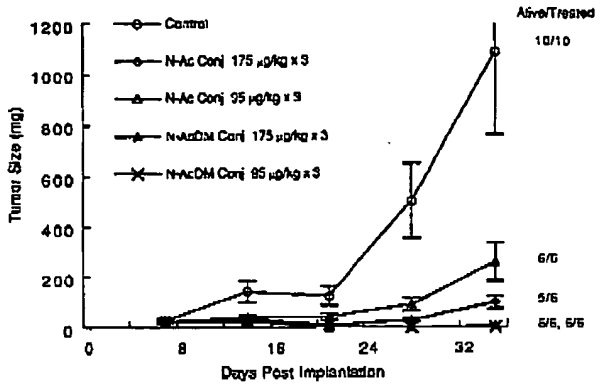

Fig. 6. A comparison of the antitumor effects of CT-M-01 conjugate of *N*-acetyl γ_{1}^{I} hydrazide and *N*-acetyl γ_{1}^{I} DM hydrazide and controls on the MX-2 xenograft tumor. Athymic mice implanted with the s.c. MX-2 breast carcinoma were treated Q4D × 3, i.p., with the two conjugates at doses of of 95 and 175 µg/kg of drug/dose × 3 doses. The *N*-acetyl-γ_{1}^{I} hydrazide and *N*-acetyl-γ_{1}^{I} DM hydrazide were included as controls and were not effective at nonlethal doses (data not shown). The *N*-acetyl-γ_{1}^{I} DM-hyd conjugate produced 6 of 6 tumor-free survivors at both doses and, as shown, were more effective that the "simple" *N*-acetyl-γ_{1}^{I} hyd conjugates. *Right*, the number of animals alive in each treatment group at day 35. In all test groups, $n = 6$; and in the control group, $n = 10$; *bars*, ± SEM for each data point.

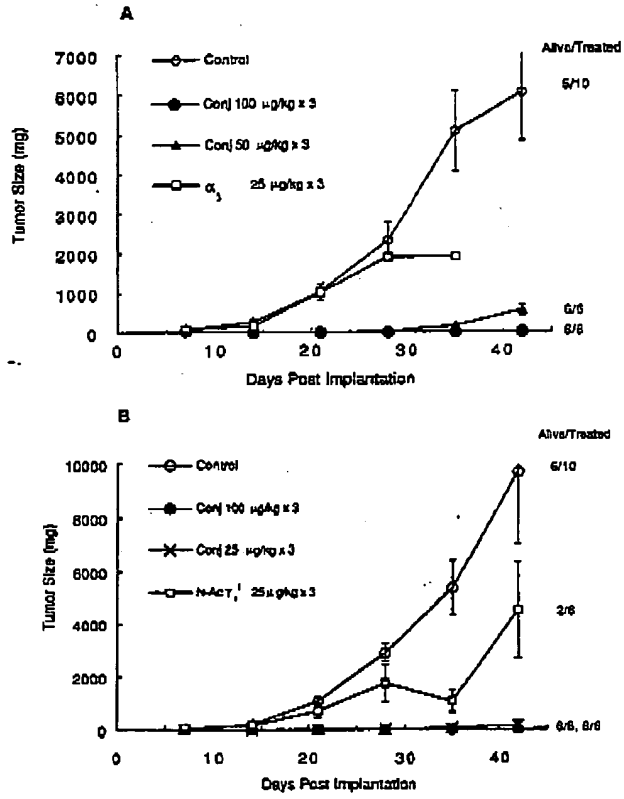

Fig. 5. Antitumor effects of a CT-M-01 conjugate of α_{3}^{I} hydrazide (*A*) and *N*-acetyl γ_{1}^{I} (*B*) and controls on the MX-1 xenograft tumor. Athymic mice implanted with the s.c. MX-1 breast carcinoma were treated Q4D × 3, i.p. with the conjugate and controls over a range of doses from 25 to 100 µg/kg of drug × 3 doses. The parent α_{3}^{I} and *N*-acetyl-γ_{1}^{I} drugs included in their respective tests as controls were lethal at doses of 25 µg/kg × 3 and above. The conjugates in both tests produced 100% tumor-free survivors at the top dose of 100 µg/kg × 3 doses. The *N*-acetyl-γ_{1}^{I} conjugate was tested i.p. or i.v. and found to give equivalent results by either route of administration. A mixture of the *N*-acetyl-γ_{1}^{I} hydrazide plus CT-M-01 at a dose of 50 µg/kg was lethal. *Right*, the number of animals alive in each treatment group at day 42. In all test groups, $n = 6$; in the control group, $n = 10$; *bars*, ± SEM for each data point.

In addition to selecting an optimal form of the drug itself, we have improved the therapeutic potential of the calicheamicin conjugates by increasing the stability of the linker. There is significant literature precedent to suggest that the introduction of steric bulk adjacent to a thiol in disulfide-based linkers effects the stability of a conjugate in serum and modulates the ease with which drug is released at the tumor site (34–36). Preliminary studies in our laboratory have suggested that the stability of calicheamicin disulfides toward reduced glutathione is proportional to the steric bulk placed adjacent to the disulfide group. These results fit with the increased therapeutic potential seen for conjugates prepared with the dimethyl linker (37). The linkage between calicheamicin and the MoAb actually contains two possible sites for drug release: the hydrazide can be cleaved by acid hydrolysis, and the disulfide bond can be cleaved by reduction. We are currently evaluating the relative importance of these two release mechanisms.

In conclusion, we have demonstrated the potential of the calicheamicins for targeted delivery. Through the process of MoAb conjugation, we have converted a potent, yet toxic series of antibiotics into effective antitumor agents, with a significant therapeutic window for treating solid tumors. Studies are in progress to establish the activity profile of both CT-M-01 and other MoAb calicheamicin conjugates in a variety of preclinical models.

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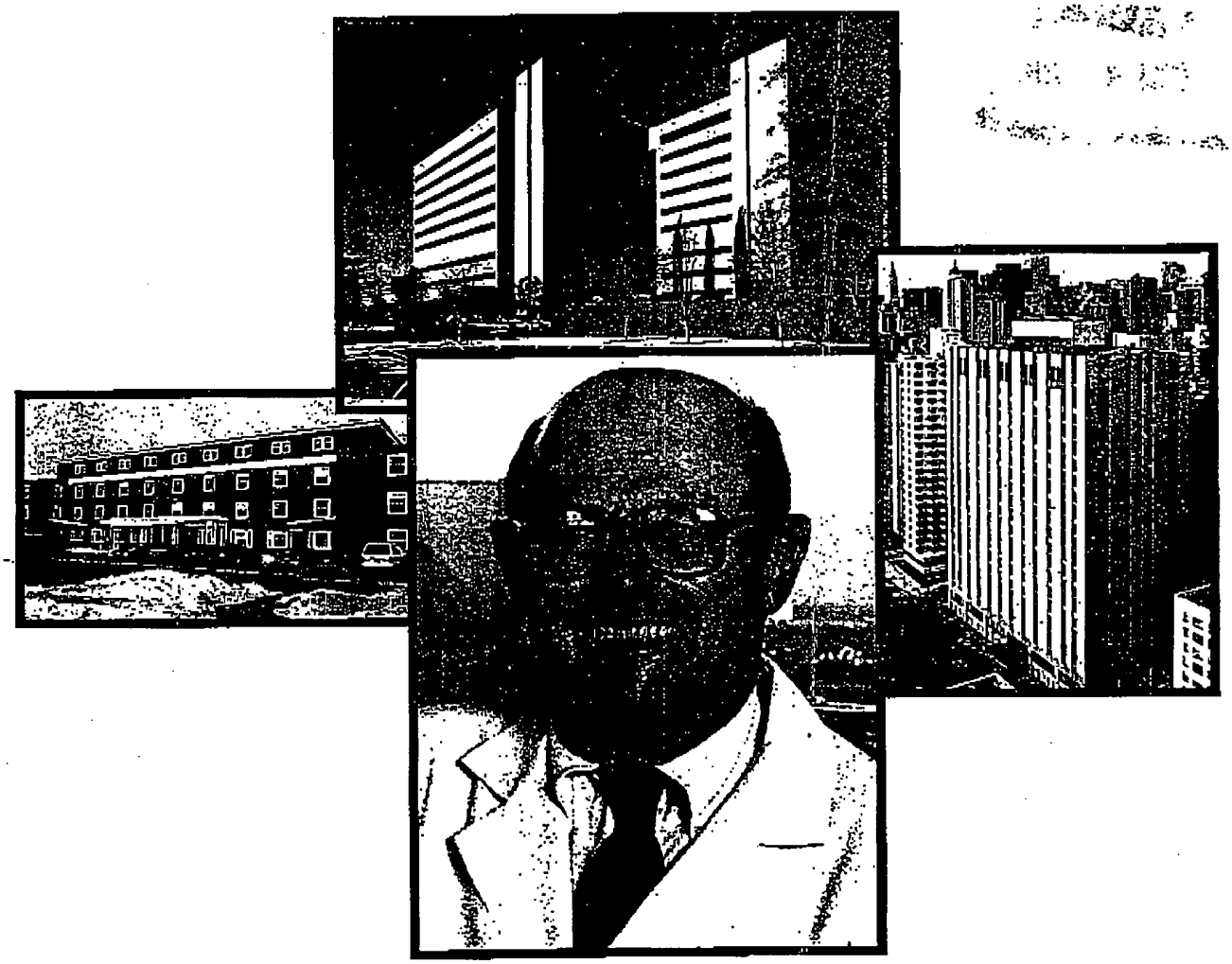

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